

Analysis of Mitotic Phosphorylation Sites in the Nuclear Pore Complex Using a MALDI LTQ Orbitrap Mass Spectrometer

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Introduction

The nuclear pore complex (NPC) is an assembly of about thirty proteins that forms the principal passageway for nucleo-cytoplasmic macromolecular traffic. In higher eukaryotes, the NPC undergoes regulated mitotic disassembly into subcomplexes, a process thought to be enacted largely via phosphorylation. In this study, we used antibodies to isolate mitotic NPC sub-complexes, and mapped mitotic phosphorylation sites in their constituent proteins. An antibody against Nup107 was used to isolate the Nup107-160 sub-complex, and monoclonal antibody mAb414 was used to isolate sub-complexes containing FG-repeat proteins involved in substrate transport through the pore. These complexes were digested and analyzed with a Thermo Scientific MALDI LTQ Orbitrap mass spectrometer to identify their constituents and to identify mitotic phosphorylation sites following phosphopeptide enrichment. 16 mitotic phosphorylation sites were identified. Nine of these occurred at sites previously annotated to be phosphorylated in large-scale screens, while seven appear to be wholly novel.

Goal

Identification of mitotic phosphorylation sites in nuclear pore (NPC) sub-complexes using MALDI LTQ Orbitrap™ MS.

Experimental Conditions

Cell culture and immunoprecipitations

HeLa cell cultures were synchronized at S-phase entry using a double-thymidine blockade. After release, the cultures were monitored for entry into mitosis and harvested by centrifugation. The pellet, containing approximately 5×10^8 mitotic cells, was frozen and then thawed in the presence of buffer containing 2% Triton X-100 and protease and phosphatase inhibitors. The cells were lysed by vigorous resuspension. For immunoprecipitation, the lysate was incubated with 200 mg of protein A-Sepharose per one milliliter of extract for 45 min at 4°C to minimize subsequent nonspecific binding to the Sepharose resin. The cleared supernatants were then incubated for 2 h at 4°C with 5 mg of mAb414 antibodies (affinity purified against Nup62) bound to protein A-Sepharose. The beads

were isolated by centrifugation for two minutes at 3000 rpm in an Eppendorf microcentrifuge and then washed extensively with PBS. The supernatant was incubated for 2 h at 4°C with 5 mg of affinity-purified anti-Nup107 antibodies bound to protein A-Sepharose. Then, the beads were isolated by centrifugation and extensively washed with PBS. Phosphatase inhibitors were included at all stages. Bound proteins were eluted by incubation with SDS-PAGE loading buffer, and stored at -80°C.

Protein digestion and phosphopeptide enrichment

One quarter of the IP samples were loaded across 2-3 wells of a 6% polyacrylamide gel (Invitrogen) and separated by SDS-PAGE. The resolved gels were stained with zinc (Bio-Rad). The top portion of the gel containing protein bands above ~70 kDa was cut into eight molecular-weight-range sections. These were destained, diced, and subjected to in-gel digestion following standard protocols. Peptides were recovered from the digested gel pieces by incubation with 3 μ l of Poros R3 resin in 5% formic acid with rotation for three hours. Following incubation, the Poros beads were removed by gel-loading pipette, and bound peptides were eluted with 70% acetonitrile. Five percent of the recovered material was spotted onto a 384-well MALDI target plate and allowed to dry, then covered with DHB matrix for subsequent analysis. The remaining recovered peptides from each band were incubated with mixing for one hour with 3 μ l of Titansphere titania beads (GL Biosciences) in the presence of 2% TFA and 150 mg/ml DHB in 70% acetonitrile. The beads were packed in a gel-loading tip using Empore C18 (3M) as a frit, washed with the same buffer solution, and then peptides were eluted with 10 μ l of ammonium hydroxide / 50% acetonitrile. The eluate was immediately added to 100 μ l of 5% aqueous TFA, and passed through a Poros R3 microcolumn made from a gel-loading tip using Empore C18 as a frit. Peptides were eluted with 2 μ l of 70% acetonitrile, 5% formic acid onto a 384-well MALDI plate and allowed to dry, then covered with DHB matrix. In parallel, one quarter of each IP sample was precipitated from sample buffer with cold acetone, resuspended, and digested with trypsin directly. The digests were subjected to the same titania purification and sample spotting procedure as for the gel bands.

Key Words

- MALDI LTQ Orbitrap
- Mitosis
- Nuclear Pore Complex
- Phosphopeptide
- Phosphorylation
- Proteomics

Mass analysis

The MALDI spots were analyzed using a Thermo Scientific MALDI LTQ Orbitrap mass spectrometer. In general, MS analysis was conducted in the Orbitrap mass analyzer using a resolution of 100k and fragmentation analysis was conducted in the LTQ ion trap. For automated data acquisition, automatic gain control (AGC), automatic spectral filtering (ASF) and the crystal positioning system (CPS) were used to automatically locate optimal sample regions. Software detection of the neutral loss of phosphate was used to automatically perform MS³ analysis of phospho-serine and threonine containing peptides. The resulting data were analyzed using Mascot™, and verified by manual inspection. Phosphopeptides failing to produce IDs in the initial search were subjected to further manual MS³ or MS⁴ (for diphosphopeptides). Data were averaged until rich spectra were obtained or no further improvement was possible.

Results and Discussion

Immunoprecipitation with anti-Nup107 or mAb414 antibodies is expected to provide purification of the NPC sub-complexes indicated in Figure 1. Analysis of the peptides recovered from the digested gel slices (Figure 2) confirmed the successful purification of these targets. In the anti-Nup107 IP, all known components of the Nup107-160 sub-complex over 70 kDa in weight were identified. Gel bands containing proteins below this weight were not

processed due to contamination with excess antibody (Figure 3). Intriguingly, several additional proteins were identified: specifically myosin 9, myosin 10, and the clathrin heavy chain (CHC). As the Sec13 protein component of the Nup107-160 complex is also a component of clathrin-coated vesicles, the slight presence of CHC may indicate some slight bridging of these complexes by Sec13. In contrast, myosin 9 was identified as the most dominant protein in the sample. While some evidence has suggested the presence of myosin in the intact nuclear pore, it is not considered a component of the Nup107-160 sub-complex, to our knowledge. Our evidence suggests tight association of myosin 9 with this sub-complex during mitosis.

In the case of the mAb414 IP, we identified all of the components of the two major expected sub-complexes, specifically those composed of Nup62/58/54 and Nup358/214/88 (Figure 4). In addition, we detected Ran-GAP1, which is known to associate with Nup358, and also SUMO1. Ran-GAP1 is also known to be modified by SUMOylation, which explains detection of this very small protein when proteins under 70 kDa would not be expected. Nuclear import proteins importin A2 and B1 were also detected. These proteins form a dimeric complex and are known to bind to FG domains during the nuclear translocation process. Two chaperone proteins were identified as well, Hsp7c and GRP78. As FG domains form natively disordered structures, these proteins may serve to stabilize these domains after NPC disassembly. Curiously,

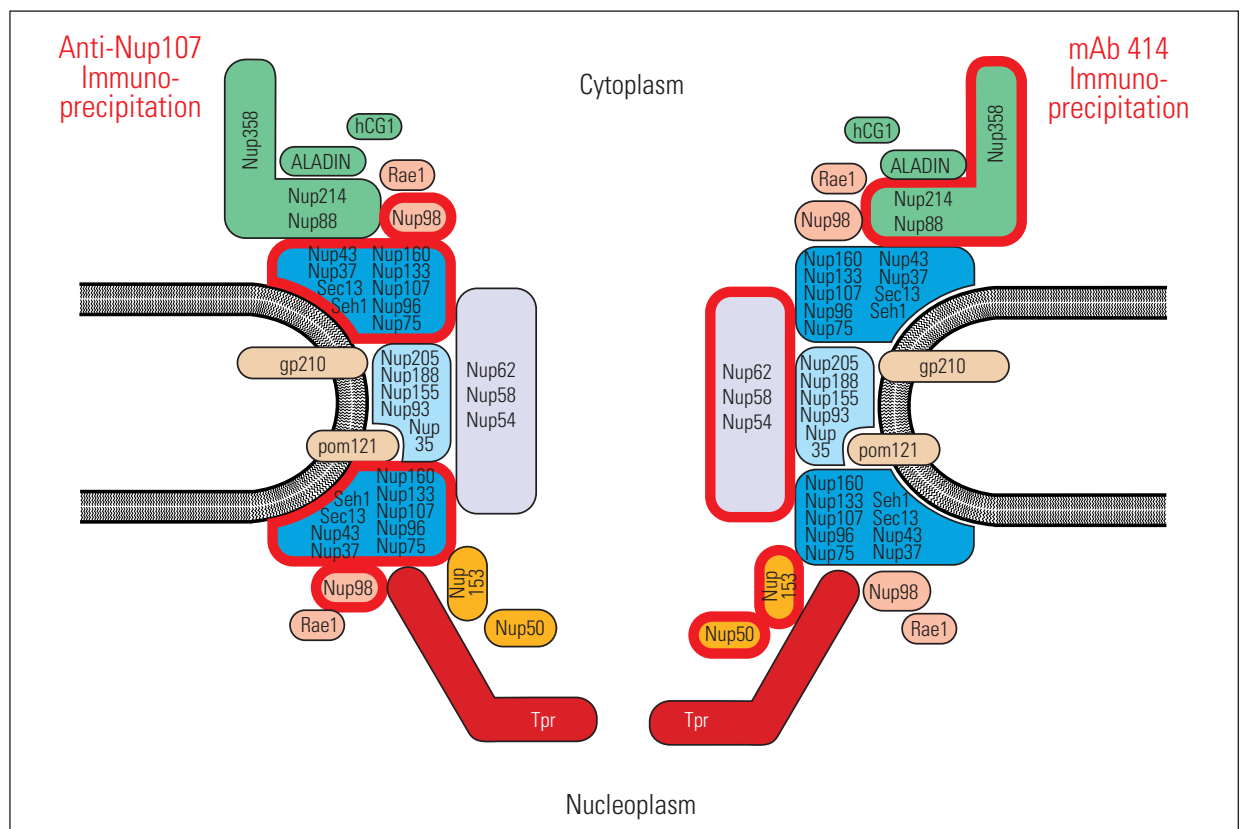


Figure 1. A schematic depiction of the assembled (interphase) nuclear pore complex (NPC). The immunoprecipitations performed in this study are expected to isolate the sub-complexes highlighted in red.

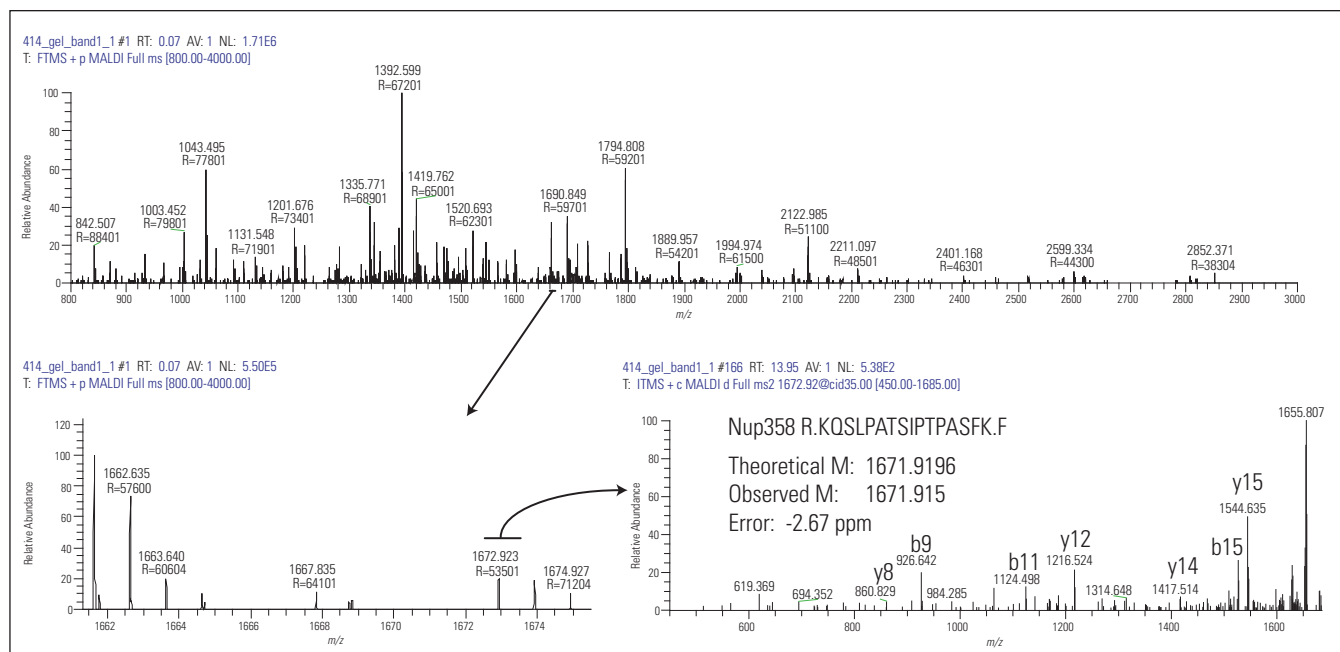


Figure 2. MALDI LTQ Orbitrap analysis of peptides recovered from a high-weight mAb414 IP gel slice. The upper spectrum shows a full MS scan at 100k (FWHM at m/z 400) resolution; 'R' indicates the observed peak resolution. At bottom left is an inset into the region 1662-1675. The minor peak at 1672.92 was automatically selected for fragmentation in the LTQ; the resulting spectrum, identified as a peptide from Nup358, is shown at the right. Calculated and observed neutral masses are indicated.

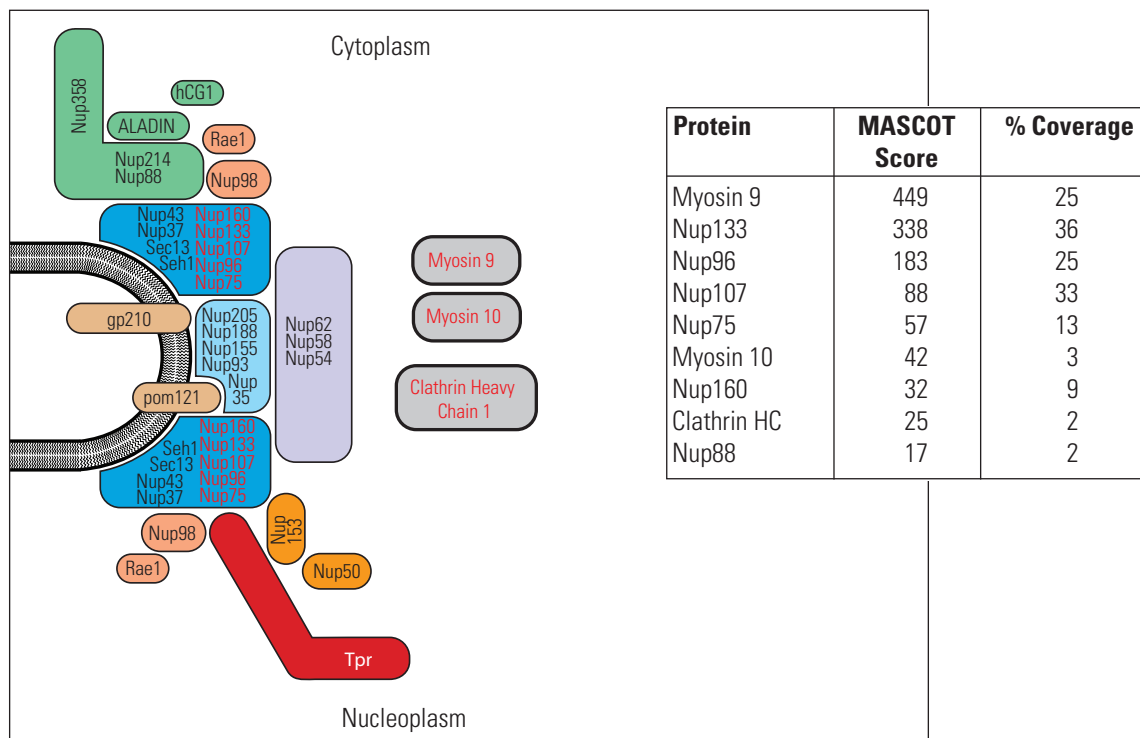


Figure 3. Proteins identified in the anti-Nup107 immunoprecipitation. Spectra obtained from all processed gel slices were searched in aggregate. All proteins in the Nup107-160 subcomplex over 60 kDa (the lower gel slice MW cutoff) were detected. Surprisingly, the most prevalent protein was Myosin 9. Myosin 10 was also observed, as well as the Clathrin Heavy Chain.

the Nup107-160 sub-complex component Nup133 was detected in this IP, and Nup96 was also tentatively identified. This seems to suggest that some fraction of Nup133 associated with the FG-domain complexes rather than Nup107-160 following NPC disassembly

Analysis of the phosphopeptide-enriched fractions (Figure 5) yielded identification of 16 phosphorylation sites in eight proteins. Nine of these sites are annotated as phosphorylated in one or more public databases (ExPASy, HPRD, Phosida) without regard to the cell cycle timing of their phosphorylation. The remaining sites appear to be wholly novel. Comparison of the anti-Nup107 data with that produced in a previous study of mitotic phosphorylation sites in the Nup107-160 sub-complex by one of us (J. Glavy, Ref 1) revealed that we identified a disparate set of phosphorylation sites, though several of the sites identified here are physically proximal to sites identified in the previous study. As we identified the significant majority of observed presumptive phosphopeptides (those giving a facile neutral loss of 98 daltons), this suggests that we failed to enrich some fraction of mitotic phosphorylation sites in the Nup107-160 sub-complex, or that perhaps there can be some variability in typical site occupancy. On a technical note, it was found that titania-based enrichment of phosphopeptides from digestion of the total IPs was significantly complicated by the presence of antibody.

This is due to the fact that immunoglobulin heavy chains are decorated with glycan structures terminated by sialic acid, and titania provides efficient purification of sialic acid-containing species. Thus, numerous antibody-derived glycopeptides were co-purified along with the phosphopeptides.

Conclusion

Immunoprecipitation afforded high-purity isolation of NPC sub-complexes from mitotic cells. Analysis of sub-complex constituents revealed the expected proteins and some surprises, such as abundant myosin 9 in the Nup107-160 complex IP, and Nup133 associated with FG-domain complexes. Phosphopeptide analysis revealed seventeen sites of mitotic phosphorylation, eight of which were wholly novel sites. MALDI analysis using an LTQ Orbitrap mass spectrometer provided rapid, high mass accuracy (<3 ppm) identification of the sub-complex protein constituents and sites of phosphorylation. The MALDI format, in combination with the sensitive MSⁿ performance of the LTQ ion trap, allowed for manual, prolonged 'revisiting' of phosphopeptides that were not successfully identified in an automated first pass, thus permitting additional identifications.

References

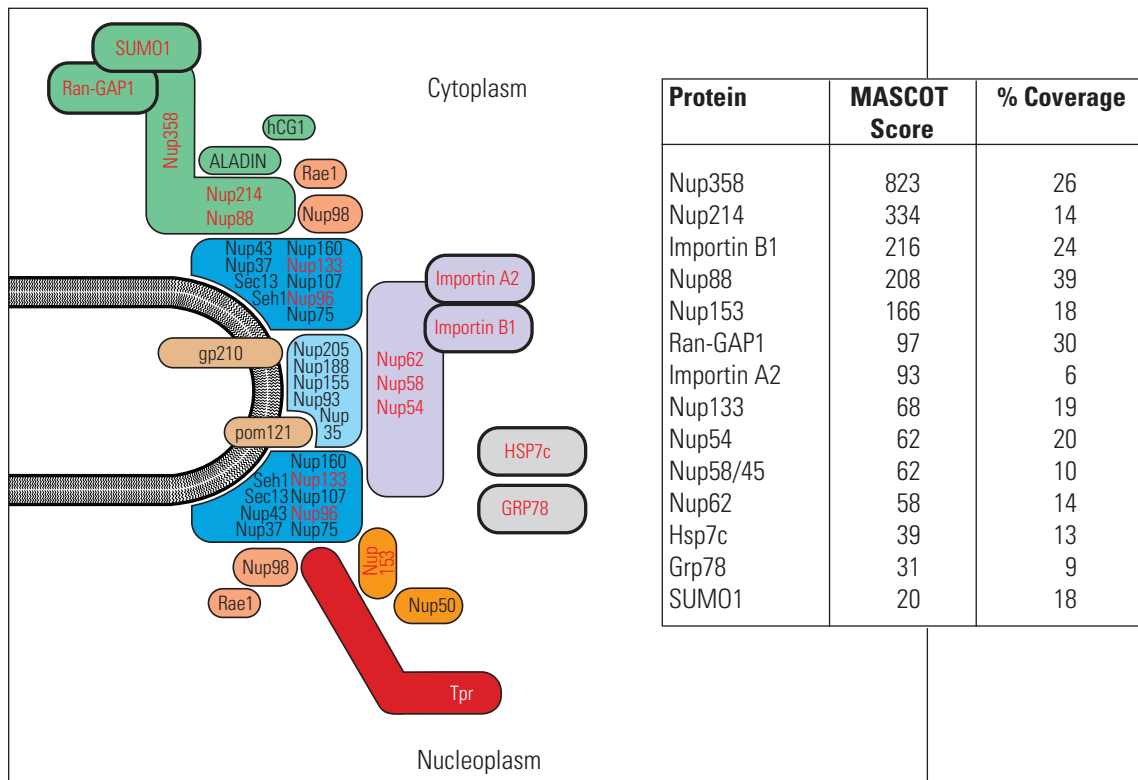


Figure 4. Proteins identified in the mAb414 immunoprecipitation. The expected Nup358/214/88 and Nup62/58/54 sub-complexes were identified, as well as Ran-GAP1 and SUMO1, which bind Nup358, and an importin A2/B1 complex, which binds FG proteins. Chaperones HSP7c and GRP78 were also identified. Intriguingly, Nup133 and Nup96 from the Nup107-160 subcomplex were also detected.

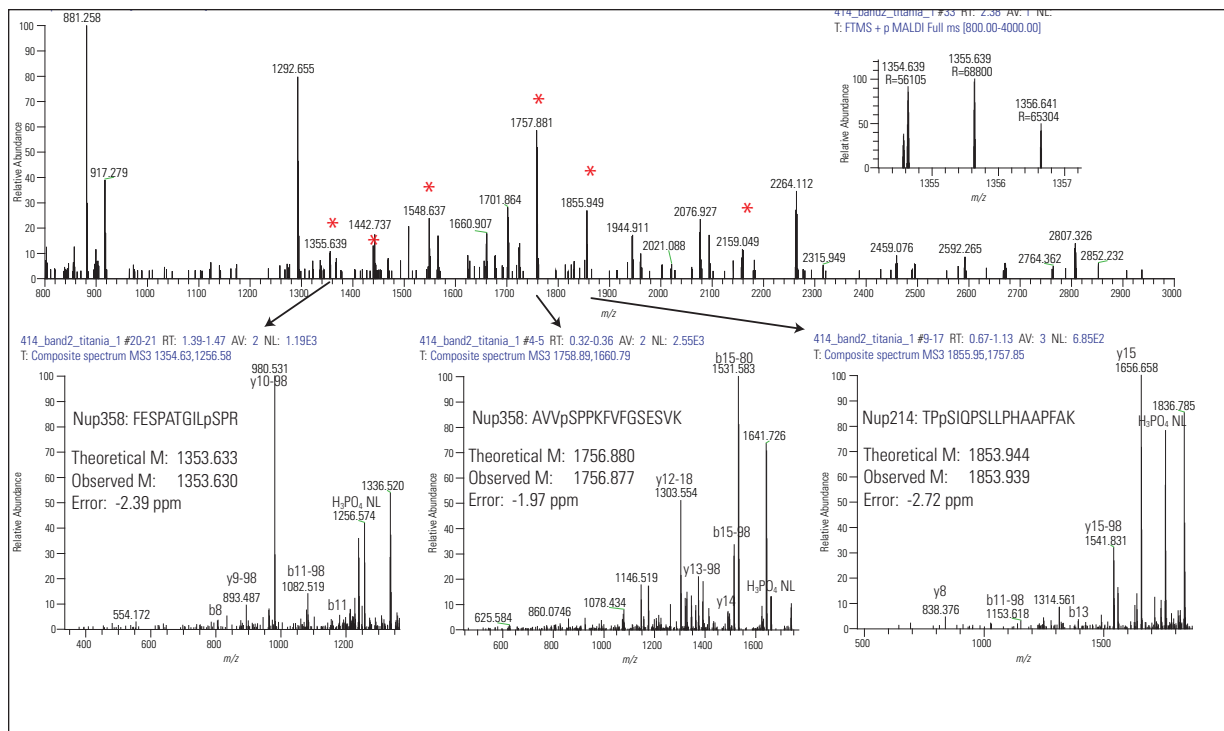


Figure 5. MALDI LTQ Orbitrap analysis of a phosphopeptide-enriched set of peptides derived from digestion of a mAb414 IP gel slice. The slice was known to contain primarily Nup358 and nup214. In the top spectrum, a full MS scan (800-4000 acquired, 800-3000 shown) is shown with phosphopeptides (as identified by facile neutral loss of 98 Da) indicated with asterisks. The inset shows resolution of an isobaric contaminant next to the phosphopeptide at 1354.63. The lower spectra show composite MS²/MS³ fragmentation spectra for the indicated phosphopeptides, acquired in the LTQ.

MAB414					
Ion	Protein	Sequence	Location	Novel?	
1284.540	Nup358	K.DSLI ¹ PHVSR.S	T2450, S2454	Known, Known	
1354.637	Nup358	R.FESPATGIL ¹ SPR.G	S948	Novel	
1548.635	Nup358	R.FGESTTGFNF ¹ SFK.S	S2270	Novel	
1676.761	Nup153	K.NTSLPPLW ¹ SPEAER.S	S209	Known	
1956.836	Importin α2	R.NVSSFPDDAT ¹ SPLQENR.N	S62	Known	
1442.748	Nup214	R.ITPPAAKPG ¹ SPOAK.S	S678	Novel	
1854.946	Nup214	R.TP ¹ SIQPSLLPHAAPFAK.S	S1023	Novel	
1311.635	Nup88	R.FFTSST ¹ SLTK.H	S168	Novel	
1323.587	Nup88	R.EDVEVAE ¹ SPLR.V	S517	Known	
1439.725	RGPD5/6/7	R.YVASVLGLT ¹ SPR.Q	S21	Known	

MAB414					
Ion	Protein	Sequence	Location	Novel?	
1896.887	Nup98-96	K.PAPPPQS ¹ Q\$PEVEQLGR.Y	S934 or S932	Novel	
1971.750	Nup98-96	K.YGLQD ¹ \$DEEEEEHPSK.T	S888	Known	
1495.684	Nup133	R.RGPLAGLGP ¹ GPR.T	S27, T28	Novel, Novel	
2128.063	Nup133	R.KGLPLGSAV ¹ SSPVLFS ¹ SPVGR.R	S45, S50	Known	
1999.963	Nup133	K.GLPLGSAV ¹ SSPVLFS ¹ SPVGR.R	S45, S50	Known	

Figure 6. Summary of phosphopeptides identified in the anti-Nup107 and mAb414 IPs. The observed ions, and their corresponding proteins, peptide sequences, and phosphorylation sites are indicated. Previously annotated and apparently novel sites are indicated.

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